Glucose Derivatives as Efficient Markers of Cell Reversible Electropermeabilization

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Abstract— **Molecules spontaneously transported inside the cells, like glucose derivatives, can also be used as electropermeabilization markers. In a previous study, we evaluated the uptake of a fluorescent deoxyglucose derivative (2-NBDG) by normal and electropermeabilized Chinese hamster cells. We extend here our previous study to two murine tumor model cells and investigate the effect of rolipram, a selective PDDE4 inhibitor, on 2-NBDG uptake by tumor cells, with or without electric pulses.2-NBDG was added to cell suspensions, and the cells exposed or not to eight square-wave electric pulses of 100-µs duration and of appropriate field amplitude delivered, were incubated at 37°C and uptake was measured by flow cytometry. In rolipram experiments, cells were similarly processed after a 15 min pre-incubation with rolipram. In spite of significant uptake of 2-NBDG, mediated by GLUT transporters into non permeabilized cells, electric pulses significantly increased the 2-NBDG uptake into both murine tumor cells, even though the electrical parameters allowing a maximal uptake were different. Pretreatment with rolipram, only at high concentrations reduced 2-NBDG uptake in non-electropermeabilized cells, affecting more severely the DC-3F cells than the LPB cells. On the contrary, rolipram treatment did not attenuated the uptake of 2-NBDG in the electropermeabilized cells. We extended to other cell lines our previous observation that glucose derivatives can be used to detect cells reversible electropermeabilization. Moreover, our data suggest that rolipram could probably be used as a tool for improving the visualization of tumor using glucose derivatives, by affecting the uptake in the surrounding normal tissue.**

Keywords— **2-NBDG, Rolipram, Electropermeabilization, Flow cytometry, Tumor cell.**

I. INTRODUCTION

Cell membrane electropermeabilization (EPN) or electroporation (EP) is a physical method that uses short and intense electric pulses to increase cell membrane permeability [1]. This method is used in molecular biology to increase the uptake of molecules such as DNA, antibodies and oligonucleotides into the cells [2]. So far, electroporation has proved to be effective as a drug delivery system to increase the uptake of nonpermeant drugs which are hydrophilic and lack transport systems (like bleomycin [3,4], or for low-permeant molecules, that poorly cross the plasma membrane [5-8]. The effectiveness of two chemotherapeutic drugs, Bleomycin and Cisplatin, in Electroporation-based therapies, like the electrochemotherapy (ECT) was demonstrated *in vitro*, *in vivo* and in clinical trials [9-15]. Several fluorescence markers are used to monitor the permeability of cells *in vitro* [16,17], all of them being nonpermeant molecules that cannot diffuse through the membrane of intact cells [8]. Even though the accumulation of data showing that electroporation as a drug delivery system is basically interesting in the case of low- or non-permeant molecules, we recently showed that glucose derivatives such as Fluorescence-labeled deoxyglucose molecules (2-NBDG), permeant molecules actively transported inside the cells, can also be used to detect cell electropermeabilization [18]. In spite of high uptake of 2-NBDG by GLUT transporters into the tumor cells, electroporation significantly increases the uptake of 2-NBDG uptake into DC-3F Chinese hamster lung fibroblasts in culture, by one to two orders of magnitude if short post-pulse incubations are performed [18]. Actually, this in vitro study was designed to explore the possibility of using the 18-FDG radioactive analogue of the glucose to assess cell electropermeabilization in vivo using Positron emission tomography imaging (PET). Rolipram, as a selective phosphodiesterase (PDE) type-4 inhibitor [19] , has been shown to decrease the uptake of radiolabeled analogues of glucose ([^{3}H] 2-DG, 14C-DG and FDG) in normal tissues such as heart and skeletal muscle [20-22]. Rolipram is a selective phosphodiesterase (PDE) type 4 inhibitor. Selective phosphodiesterase (PDE4) enzyme has been proposed as a means of reducing the radiolabeled deoxyglucose molecule (FDG or 14C-DG) uptake in normal tissue com-

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pared to tumor tissue [21,22] which can improve the visualization of tumor or inflammatory lesions by PET using FDG *in vivo*. The principal function of Rolipram could rely on the decrease in hexokinase activity in normal tissues (Kobayashi et al. 2002). It was concluded that the radioactivity ratio in tumor tissue is improved after treating with rolipram as this inhibitor does not affect the tumor tissue as much as normal tissue [21], that the experiments would then be transferred in preclinical using. Therefore, to use PET/FDG *in vivo* to map the electroporation effect of ECT, based on the potentialities revealed *in vitro* by the fluorescent analogue of glucose 2-NBDG in our previous study, we had to be extend our observations to other tumor cell types *in vitro*. The aims of the present work were: (i) to confirm the results of our first study in the LPB murine fibrosarcoma and B16-F10 murine melanoma models, and (ii) to evaluate the effect of rolipram on the 2-NBDG uptake in electropermeabilized and non permeabilized cells.

II. MATERIALS AND METHODS

A. Tumor Cells and Preparation of Rolipram

Three tumor cell lines were used, the murine fibrosarcoma LPB cells, the murine melanoma B16F10 cells and the spontaneously transformed Chinese hamster DC-3F fibroblasts. They were grown in Minimum Essential Medium (SMEM) supplement with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin solution (all from Life Technologies, California, USA). Cells were detached with trypsin /EDTA (Life Technologies), counted and seeded in new flasks using standard protocols.

Rolipram ((RS)-4-(3-cyclopentyloxy-4-methoxy-phenyl) pyrrolidin-2-one) was purchased from Sigma-Aldrich, (Lyon, France) and suspended in saline normal to give final concentrations of 30 µM, 10 µM, 30 µM and 100 µ M.

B. Cell Electropermeabilization and Flow Cytometry

After After trypsination, cells were centrifuged for 10 min at 1000 rpm at 4˚C and suspended in phosphate-buffered saline (PBS) to obtain $5*10^5$ cells per 42.5 µL (~10^7 cells/ml). For each experiment, 5 µl of 2-NBDG (MW=342.26g/mol) (Molecular Probes, Eugene, OR) were added to the 42.5 µl of the cell suspension. Immediately after, the cells in the presence of the NBDG were placed in a sterile electroporation cuvette (Cell Projects Ltd, Kent UK, with 1 mm gap between the electrodes). Eight square-wave electric pulses of appropriate voltage and of 100-µs duration were delivered at a repetition frequency of 1 Hz or 5 kHz using a Cliniporator (IGEA, Carpi, Italy). In competition experiments, after trypsinization, cells were suspended in PBS containing 5.5mM D-glucose (Merck, Darmstadt, Germany). In rolipram experiments, after 15 min incubation with rolipram, 2-NBDG was added to the cells. All experiments were performed at 37˚C. In all the experiments, 2-NBDG uptake was stopped by diluting the cells with 15 ml of pre-cooled phosphate buffered saline (PBS). Cells were subsequently resuspended in 500-µl pre-cooled phosphate buffered saline (PBS) and maintained at 4 ˚C before flow cytometry analysis. For each measurement, fluorescence was measured with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were excited at 488 nm and the emitted 2-NBDG fluorescence collected at 640 nm. Data from each flow cytometric measurement (Relative scattered light or fluorescence intensity of each event) were stored under FCS (Flow Cytometry Standard) Format. Controls (no 2-NBDG, and no pulse in the presence of 2-NBDG) were included in all the experiments.

C. Data Processing

All experiments were repeated at least three times on different days. Results are presented as the relative difference in the peak channel of the fluorescence intensity between each group and the corresponding control performed in the same experiment. Results are also presented as the percentage of events in the fluorescent cells exposed to 2-NBDG, or in the most fluorescent part of the cell population exposed to 2-NBDG and to the electric pulses (which should correspond to the percentage of reversibly permeabilized cells, detected by the high 2-NBDG uptake). ANOVA (one way or three ways) and independent student t-test were used to evaluate the statistical significance of the differences between the experimental and the control groups. A level of $p\text{-value}<0.05$ was considered significant.

III. RESULTS

A. Effect of Electroporation on 2-NBDG Uptake

2-NBDG uptake was studied in the absence or presence of D-glucose as a function of the electric pulses amplitude in the LPB and B16F10 cells. *LBP cells* were pulsed immediately after the addition of 2-NBDG using electric pulses with amplitudes of from 900 V/cm to 1500 V/cm. After the pulses cells were incubated at 37˚C for 10 min. While fluorescence distribution was unimodal in the case of cells exposed only to the 2-NBDG, distribution was bimodal if the cells were also exposed to the electric pulses. Therefore, two parameters were considered to analyze the effects on the electric pulses on 2-NBDG uptake: the percentage of permeabilized cells that is the percentage of cells in the part of the cell population which display high fluorescence intensity, and the level of 2-NBDG uptake by the reversibly electropermeabilized cells (the irreversibly permeabilized cells will not be able to maintain a high content of 2-NBDG, which will be lost during the washing procedures).